Locating the barnacle settlement pheromone: spatial and ontogenetic expression of the settlement-inducing protein complex of *Balanus amphitrite*

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Barnacles are prominent members of hard substratum benthic communities and their study has been important to advances in experimental ecology and contemporary ecological theory. Having recently characterized the cue to gregarious settlement of *Balanus amphitrite*, the settlement-inducing protein complex (SIPC), we use two polyclonal antibodies to examine the tissue distribution and ontogenetic expression of this glycoprotein. These antibodies were raised against two separate peptides located near the N- and C-termini of the SIPC and were used to detect the glycoprotein by western blotting and immunohistochemistry. By *in situ* hybridization we also show that the SIPC mRNA co-occurs with the expressed glycoprotein in the cuticles of *both* nauplius and cypris larval stages and the adult. In the larvae, the SIPC is expressed most strongly in the mouthparts and the hindgut of the stage 2 nauplius and in the thoracopods, antennules and bivalved carapace of the cyprid. In adult *B. amphitrite*, the expressed SIPC is present in protein extracts of the shell and in all organs that are lined by cuticular tissues. We suggest that the SIPC is produced by the epidermal cells that secrete the cuticle and discuss these observations with regard to earlier studies and the role of the SIPC as a contact pheromone.

Keywords: arthropod; *Balanus amphitrite*; barnacle; pheromone; larval settlement; settlement-inducing protein complex

1. INTRODUCTION

The colonization of surfaces by benthic marine organisms is mediated by *both* physical and chemical processes. Increasingly, there is evidence of the importance of biogenic chemical cues to surface colonization (see for example, Pawlik 1992; Hadfield & Paul 2001; Steinberg *et al.* 2001 for reviews). The difficulties associated with identifying such cues have been described (Zimmer & Butman 2000; Hadfield & Paul 2001; Steinberg *et al.* 2001). As a result only one inductive cue has been fully characterized in an ecological context, namely histamine, which induces settlement of the sea urchin, *Holopneustes purpurescens*, on host algae (Swanson *et al.* 2004). Likewise, the inhibition of surface colonization of algae by halogenated metabolites (Steinberg *et al.* 2001; Paul *et al.* 2006) are the only cases, to our knowledge, where inhibitory compounds have been shown to be released at their site of action at environmentally realistic concentrations.

The underlying chemical ecology of the gregarious settlement behaviour of barnacles is of central interest to behavioural and community ecologists due to the important role of barnacles in the structuring of marine communities (Leslie 2005; Leslie *et al.* 2005). Moreover,

barnacles are major fouling organisms of ship hulls and other marine structures (Knight-Jones & Crisp 1953), so there is also an economic interest in understanding their settlement behaviour. Gregarious settlement in barnacles involves a specific behavioural response by the free-swimming cypris larva to *both* physical and chemical characteristics of the substratum as well as conspecific biogenic cues (Knight-Jones 1953; Pawlik 1992; Clare & Matsumura 2000). We have recently characterized a cue to gregarious settlement of *Balanus amphitrite*, a pheromone known as the 'settlement-inducing protein complex' (SIPC; Matsumura *et al.* 1998a), showing it to be a novel glycoprotein similar to thioester proteins (Dreanno *et al.* in revision). Earlier studies of the corresponding cue of *Semibalanus balanoides* suggested that an uncharacterized factor, 'arthropodin', associated with the barnacle epicuticle, induced settlement of the cyprid (Knight-Jones & Crisp 1953; Crisp & Meadows 1962; Larman *et al.* 1982). The SIPC and arthropodin, which are active when surface bound, may be synonymous but were isolated by different protocols. While the results of bioassays suggested that the SIPC is present in tissues at ecologically realistic concentrations (Dreanno *et al.* in revision), it has yet to be ascertained whether the cue is expressed at the adult's surface where it can be contacted by cyprids. Some progress has already been made towards this goal. The SIPC of *B. amphitrite* has been shown to be expressed in the larval stages and in the adult soft body tissues and shell (Matsumura *et al.* 1998b) and the SIPC mRNA is present during larval development and in the cirri, egg mass, and

penis of the adult (Dreanno *et al.* in revision), all of which comprise cuticle. In a related article (Dreanno *et al.* 2006) we have shown that the cyprid adhesive, a putative tegumental secretion that induces settlement, is related to the SIPC.

Here, we investigate the tissue distribution of the SIPC cue at the gene and protein levels in different developmental stages to understand its ontogenetic expression and role. We use *in situ* hybridization of a SIPC mRNA probe and immunohistochemistry using separate antibodies raised to the N- and C-terminal regions of the *B. amphitrite* SIPC, to demonstrate that this glycoprotein is strongly expressed in certain appendages of each larval stage, in the hind gut of the nauplius, the cuticle of the cyprid's bivalved carapace and in the cuticle of the adult. Our results, which demonstrate the co-occurrence of SIPC mRNA and the SIPC glycoprotein, provide strong support for the earlier hypothesis that the gregarious settlement cue of barnacles is a cuticular protein (Knight-Jones 1953; Crisp & Meadows 1962).

2. MATERIAL AND METHODS

(a) *Collection of* B. amphitrite

Samples of adult *B. amphitrite* were collected from Lake Hamana (Japan) and Beaufort (North Carolina, USA) and maintained in the laboratory until they were required for experiments. Larvae of *B. amphitrite* were cultured in the laboratory from the adult brood stocks according to Vogan *et al.* (2003).

(b) *Synthesis of the two* B. amphitrite *settlement-inducing protein complex antibodies*

Two peptides were designed to putative epitopes near the N- and C-terminal regions of the SIPC protein (see Dreanno *et al.* (in revision) for the complete sequence); these were H_2N-C + STHKKYESHVKTEF-$CONH_2$ (amino acid positions 424–437) and H_2N-C + PEERNI-QEYELTPAA-COOH (amino acid positions 1533–1547), respectively (figure 1a). Each peptide was coupled to bovine serum albumin and used to generate separate polyclonal anti-SIPC antibodies in rabbit (Eurogentec); we called these SIPC-N and SIPC-C subsequently to reflect the peptides located in the N- and C-terminal regions, respectively, of the SIPC. Each antibody was purified by affinity chromatography by coupling the antigenic peptide to a Thiopropyl-Sepharose matrix. The specificity of each antibody to the *B. amphitrite* SIPC was confirmed by immunoprecipitation and then immunoblotting using the polyclonal antibody raised against the 88 kDa SIPC subunit, which cross-reacts with all the SIPC subunits seen on SDS-PAGE (Matsumura *et al.* 1998a). The species-specificity of each antibody was finally determined by immunoblotting against crude protein extract from *Balanus improvisus*, *Megabalanus rosa* and *Elminius modestus*. Samples for immunoblotting were, in each case, reduced and denatured in SDS-PAGE sample buffer (Laemmli 1970) at 100 °C for 10 min and then electrophoresed on 7.5% polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membrane using 10 mM CAPS buffer at pH 11 and 10% methanol. The membranes were then rinsed with saline Tris–buffer (TBS; 0.5 M NaCl, 20 mM Tris–HCl, pH 7.8), blocked for 1 h at room temperature with 5% skimmed milk in TBS
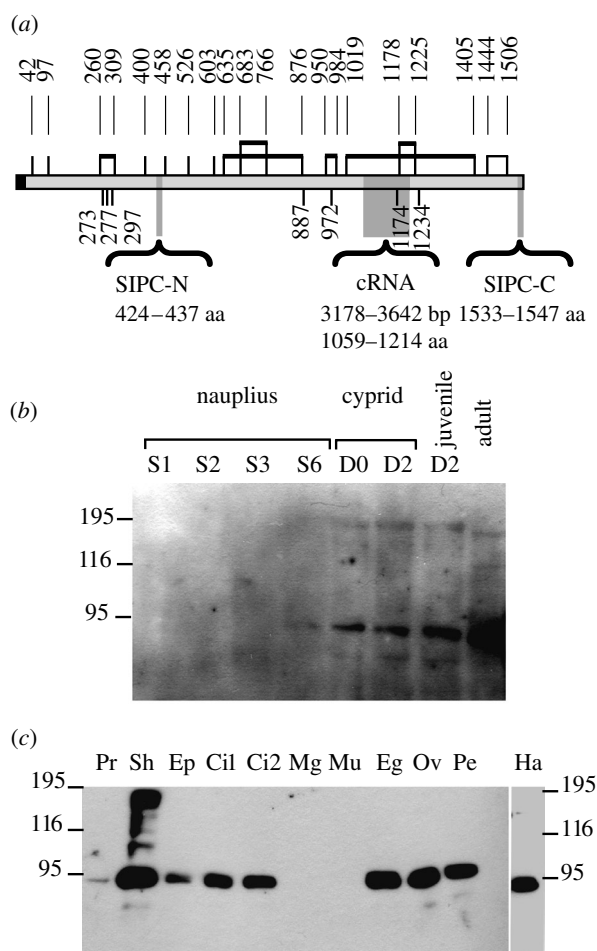

Figure 1. Location of the SIPC antigenic peptides and cRNA probe together with the western blot analysis of the expression pattern of the *B. amphitrite* SIPC. (*a*) Location of the peptides in the mature SIPC protein that were used to generate the SIPC-N and SIPC-C polyclonal antibodies and the location of the cRNA probe (given in bp and aa from the start codon; figure adapted from Dreanno *et al.* (in revision)). (*b*) Expression of the SIPC protein in different developmental stages. Protein from each sample (5 μg) was immunoblotted with anti-SIPC ($n=1$ experiment). Abbreviations: nauplius, S1, stage 1 nauplius; S2, stage 2 nauplius; S3, stage 3 nauplius; S6, stage 6 nauplius; cyprid D0, cyprid on day of moult from nauplius stage 6; D2, cyprid 2 days after moult; juvenile D2, juvenile 2 days post-metamorphosis from the cyprid. (*c*) Tissue distribution of the expressed SIPC in adult tissues. Protein from each sample (10 μg) was immunoblotted with the anti-SIPC antibody. Results are representative of three independent experiments performed using pooled adult barnacle tissues. Abbreviations: Ci1, cirri (endopod and exopod); Ci2, cirri (protopod); Eg, egg mass; Ep, epidermis; Ha, haemolymph; Mg, midgut; Mu, muscle; Ov, ovary; Pe, penis; Pr, prosoma (soft body); Sh, shell.

containing 0.1% Tween 20 (TTBS) and incubated overnight with *either* the SIPC-N or SIPC-C antibodies diluted in TTBS, containing 2.5% skimmed milk and 0.01% NaN_3 at 4 °C. After rinsing with TTBS, the membranes were incubated with the secondary antibody (1 : 5000 dilution, HRPO-conjugated anti-rabbit IgG goat antibody (Chemicon)) and immunoreactive bands were detected using the Lumi-light chemiluminescence kit (Roche). The blots were then silver stained to determine the amount of protein loaded on the gel and the transfer efficiency (Jacobson & Karsnas 1990).

(c) *Expression of the settlement-inducing protein complex in* B. amphitrite *determined by western blotting*

The expression of the SIPC in adult *B. amphitrite* was investigated in the shell, the prosoma and separately in the epidermis, cirri, midgut, muscle tissue, egg mass, ovary, penis and haemolymph by western blotting.

Adult tissues were dissected under a binocular microscope and rinsed with an ice-cold Ringer's solution (430 mM NaCl, 10 mM KCl, 10 mM CaCl$_2$, 50 mM MgCl$_2$, 10 mM Tris, pH 8.0) supplemented with protease inhibitors (complete EDTA-free protease inhibitor cocktail (Roche)) and 2 mM EDTA. Samples of haemolymph (approx. 20 µl per barnacle) were obtained by piercing the adult shell near the base with a hypodermic needle and removing a sample of some haemolymph by syringe. Special care was taken to avoid penetrating the mantle cavity, which would have diluted the haemolymph with seawater. The haemolymph was then centrifuged at 1300 g for 10 min at 4 °C and the supernatant was used in the analyses. The expression of the SIPC in whole larvae (from a single batch) was determined in nauplius stages 1, 2, 3 and 6, in the cyprid immediately after the moult from the sixth-stage nauplius (D0) and at 2 days post-moult (D2), and in newly settled juveniles (2 days after settlement). Laboratory-reared *B. amphitrite* larvae were collected by filtration, washed twice with artificial seawater and then frozen with liquid nitrogen and stored at −80 °C until analysis. Adult and larval samples were homogenized in a RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% DOC, 0.1% SDS, 50 mM Tris–HCl, pH 8) supplemented with protease inhibitors (complete EDTA-free protease inhibitor cocktail) and the expression of the SIPC in the tissue extracts was determined by western blotting. Five micrograms per sample were loaded onto the gel. At the end of the experiment, the blot was silver stained to determine: (i) whether the amount of protein loaded in each well was the same and (ii) the efficiency of transfer.

(d) *Detection of the* B. amphitrite *settlement-inducing protein complex mRNA by* in situ *hybridization*

The *B. amphitrite* larvae (stage 2 nauplii and cyprids) or adults were anaesthetized in a menthol bath (a small hole was made near the base of the shell of the adults to increase exposure) and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C for 36 h; samples to be sectioned were then dehydrated and embedded in paraffin wax (Paraplast Plus, Kendall) and 7 µm sections were cut and mounted on Superfrost Plus slides. The presence of the *B. amphitrite* SIPC mRNA transcript in whole-mount samples or tissue sections was then detected using Digoxigenin (DIG) labelled antisense and sense cRNA probes (DIG RNA labelling kit, Roche). The *in situ* hybridization protocols for whole-mount and tissue sections followed methods described by Jowett (2001) and Nakamoto *et al.* (2005), respectively. The DIG labelled probes were synthesized from a 465 bp SIPC cDNA fragment (nucleotide position 3178–3642, amino acid position 1059-1214) (figure 1*a*) and verified on a formaldehyde agarose gel; probes were kept at −80 °C until use. An anti-DIG antibody conjugated to alkaline phosphatase was used at a 1 : 5000 dilution (stock concentration 0.75 U µl^{-1}) to detect the hybridized cRNA probes. The signal was then developed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl

phosphate (BCIP; Roche) diluted in a detection buffer (100 mM NaCl, 50 mM MgCl$_2$, 0.1% Tween 20, 1 mM levamisol, 100 mM Tris–HCl pH 9.5); the reactions were stopped by washing the larvae or the slides with detection buffer. Larvae were post-fixed, dehydrated and mounted on glass slides. Digital photographs were taken with a Canon *PowerShot G5* camera attached to a Zeiss Axioskop-2 microscope.

(e) *Detection of expressed settlement-inducing protein complex in* B. amphitrite *by immunohistochemistry*

Immunohistochemistry was performed on *both* tissue sections and whole-mount specimens of *B. amphitrite*. For tissue sections, cyprids and adults were anaesthetized, fixed for 2 h at 4 °C, dehydrated, embedded and sectioned as described above. Dewaxed 7 µm sections were then rehydrated, immersed in 10 mM sodium citrate buffer (pH 6.0) and steamed for 20 min. The sections were then rinsed with PBS and incubated for 1 h at room temperature with a blocking solution (5% goat serum, 0.5% Tween 20 in PBS, pH 7.2). The sections were then incubated in a moist chamber with *either* the SIPC-N or SIPC-C antibodies in the blocking solution at 4 °C overnight. The sections were then blocked with 3% H$_2$O$_2$ in PBS for 15 min, incubated with the secondary antibody (HRPO-conjugated anti-rabbit IgG goat antibody used at a 1 : 500 dilution (Chemicon)), developed with AEC chromogen and counterstained with haematoxylin QS (Vector). Deparaffinized sections were also stained with Masson's trichrome (Martoja & Martoja-Pierson 1967) for histological studies. Whole-mount immunohistochemistry was performed on anaesthetized cyprids that were fixed initially in 4% paraformaldehyde in PBS at 4 °C for 1 h and then in cold methanol (−20 °C) for 15 min. Following fixation the cyprids were washed three times with PBT buffer (PBS, 0.5% Tween 20) and then blocked and incubated with the SIPC-N and SIPC-C antibodies as above. The cyprids were then washed with PBT and incubated with the secondary antibody (1 : 500 dilution, alkaline phosphatase conjugated anti-rabbit IgG goat antibody (Jackson ImmunoResearch)) for 1 h at room temperature. The reaction was developed with BCIP and NBT as above. Controls were performed for all the experiments by substituting the primary antibody with the non-immune serum. Photomicrography was performed as above.

3. RESULTS
(a) *Specificity of the settlement-inducing protein complex-N and settlement-inducing protein complex-C polyclonal antibodies*

The anti-88 kDa SIPC polyclonal antibody (Matsumura *et al.* 1998*b*) recognized the SIPC-N and SIPC-C immunoprecipitated proteins on a SDS-PAGE gel (figure 1*a* in electronic supplementary material). The SIPC-N and SIPC-C antibodies also recognized 90 and 190 kDa putative SIPC bands (figure 1*b* in electronic supplementary material); these molecular masses agreed with expected values (Matsumura *et al.* 1998*a*). SIPC-N and SIPC-C were species-specific as they did not cross-react with the crude protein extracts from allospecific barnacles (figure 1*b* in electronic supplementary material).
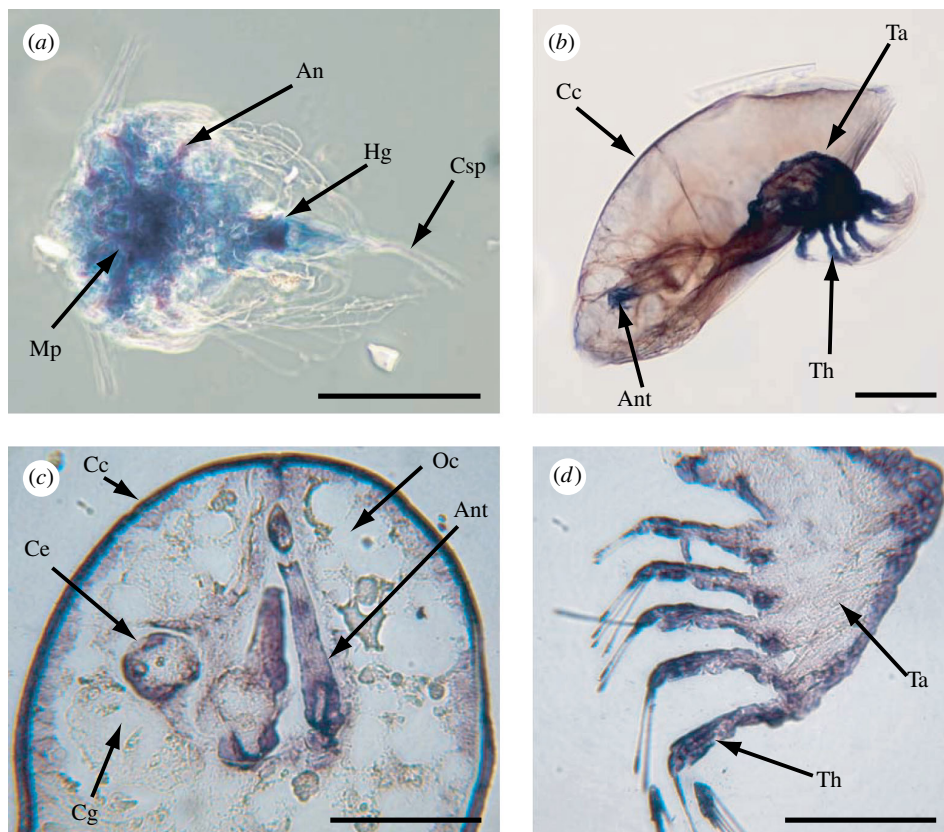
Figure 2. Expression of the SIPC mRNA transcript in *B. amphitrite*. A blue–purple staining indicates the presence of the SIPC mRNA transcript. (*a*) Dorsal view of a stage 2 nauplius. (*b*) Lateral view of a cyprid. (*c*) Sagittal section of a cyprid in the region of the antennules. (*d*) Sagittal section of a cyprid showing the thoracopods. Results are representative of three independent batches of larvae with a minimum of 50 larvae for each stage. Abbreviations: An, antennae; Ant, antennule; Cc, cyprid bivalved carapace cuticle; Ce, compound eye; Cg, cement gland; Csp, caudal spine; Hg, hindgut, Mp, mouthparts; Ta, thorax; Th, thoracopod; Oc, oil cells. Scale bars = 100 μm.
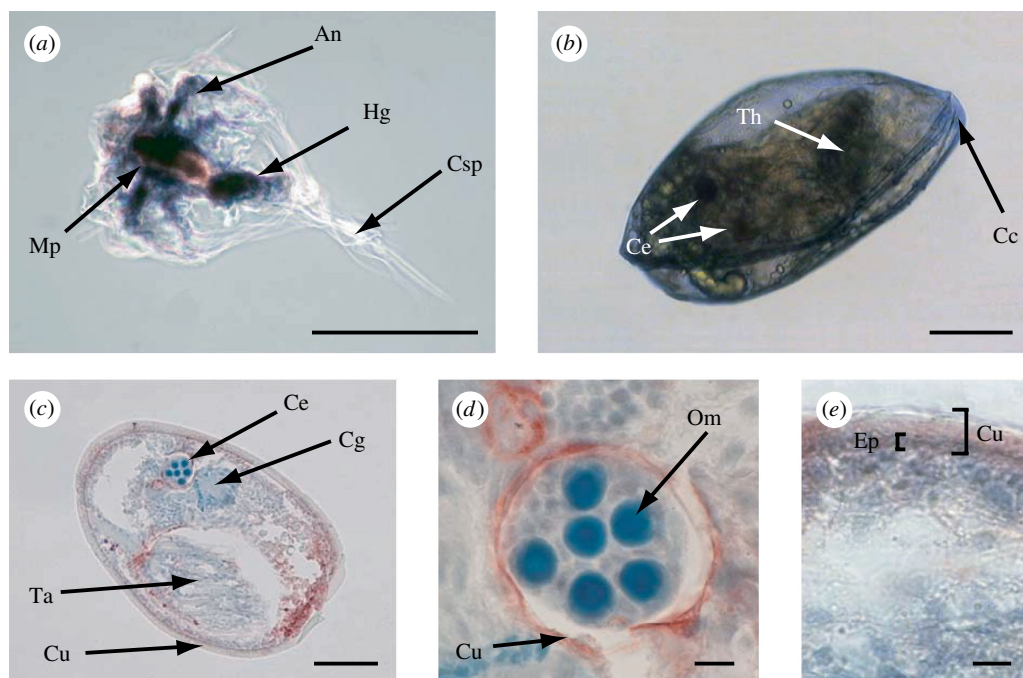

Figure 3. Expression of the SIPC protein in *B. amphitrite* larvae. A blue–purple staining reaction indicates the presence of the expressed SIPC in the whole-mount larvae. An orange–red staining reaction indicates the presence of the expressed SIPC in cyprid sections. (*a*) Dorsal view of a stage 2 nauplius. (*b*) Dorso-lateral view of a cyprid. (*c*) Sagittal section of a cyprid. (*d*) Detail of the compound eye showing the positively stained cuticle (the basophilic ommatidia are counterstained blue). (*e*) Detail of the cyprid cuticle. Results are representative of three independent batches of larvae and used a minimum of 50 larvae for each stage. Abbreviations: An, antennae; Cc, cyprid bivalved carapace; Ce, compound eye; Cg, cement gland; Csp, caudal spine; Cu, cuticle; Ep, epidermis; Hg, hindgut; Mp, mouthparts; Om, ommatidium; Ta, thorax; Th, thoracopods. Scale bars *a*–*c* = 100 μm; *d* and *e* = 10 μm.
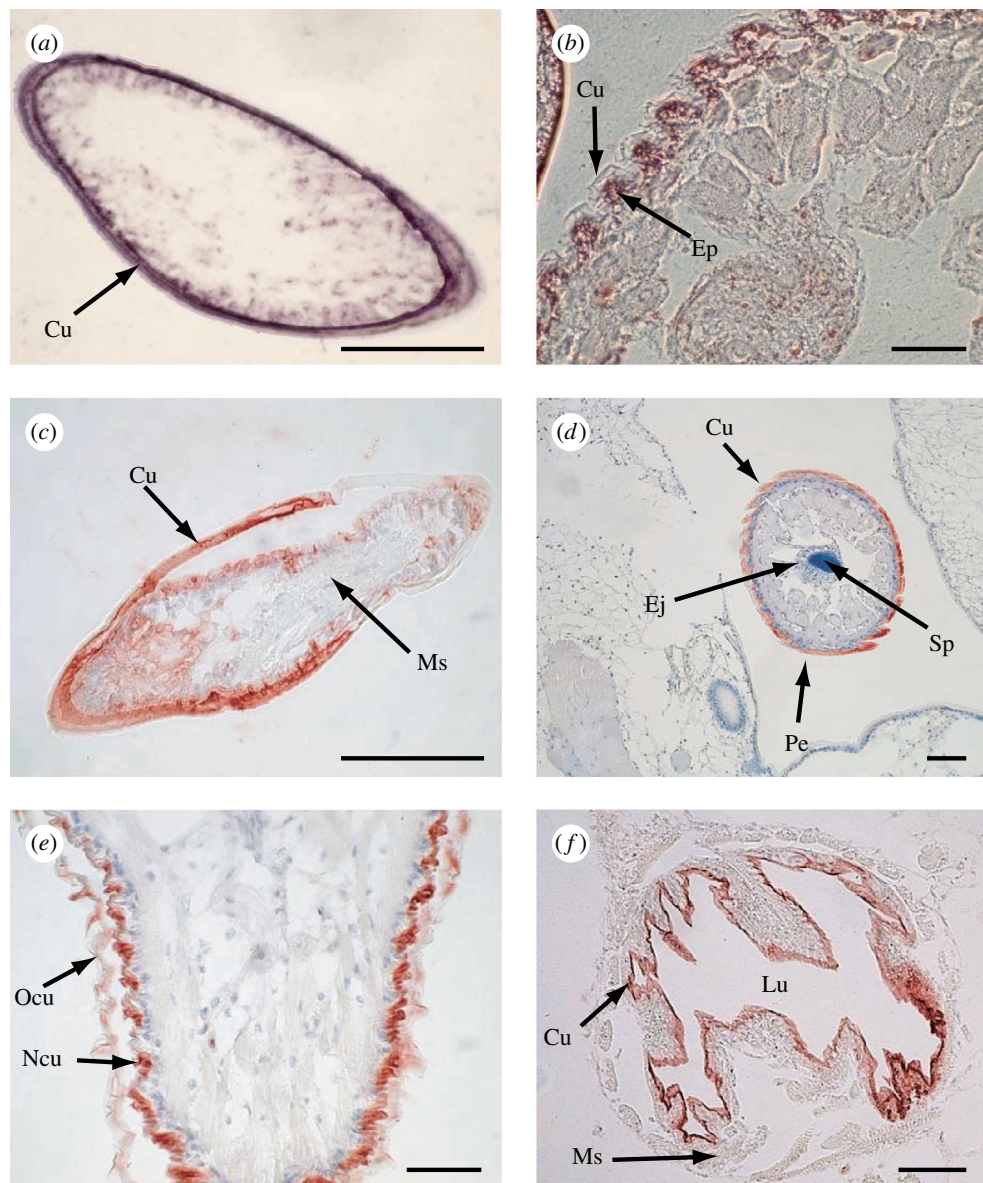
Figure 4. Distribution of the SIPC mRNA (*a* and *b*) and expressed protein (*c–f*) in adult *B. amphitrite*. A blue-purple staining indicates the presence of the SIPC mRNA transcript. An orange–red staining indicates the presence of the expressed SIPC. (*a*) Sagittal section of a cirrus to show the intense staining reaction. (*b*) Transverse section of the penis to show staining of the epidermis. (*c*) Sagittal section of a cirrus showing the localized staining of the cuticle associated with the expressed SIPC. (*d*) Transverse section of the penis showing the cuticular SIPC. (*e*) Detail of the cuticle of the penis showing staining associated with both the old and new cuticle. (*f*) Transverse section of the hindgut showing intense staining of the cuticle. Results are representative of three replicate experiments, each with six adults. Abbreviations: Cu, cuticle; Ej, ejaculatory duct; Ep, epidermis; Lu, lumen of hindgut; Ms: striated muscles, Ncu, new cuticle; Ocu, old cuticle; Pe, penis; Sp, sperm. All scale bars 100 μm except *b* and *e* = 50 μm.

(b) *Ontogeny and tissue distribution of the expressed settlement-inducing protein complex in* B. amphitrite

The expressed SIPC was detected first in the stage 6 nauplius and peaked in the adult barnacle (figure 1*b*). Figure 1*c* shows the tissue distribution of the SIPC in adults. The SIPC was expressed in the shell fraction, the haemolymph and all the adult body tissues examined except for muscle tissue and the midgut.

(c) *Distribution of the settlement-inducing protein complex mRNA and the expressed settlement-inducing protein complex in larval* B. amphitrite

Figure 2*a* shows the localization of the SIPC mRNA in the stage 2 nauplius where a strong reaction was observed in

the region of the mouthparts, segmented limbs (antennae and mandibles) and the hindgut. Similar results were obtained for stage 1 and 3 nauplii. In the cyprid, the SIPC mRNA was found in the epidermis underlying the bivalved carapace (figure 2*c*), in the thoracopods (figure 2*b,d*), in the cuticular folds at the base of each antennule (figure 2*b,c*) and in the outer surface of the compound eyes, which are covered with a thin layer of cuticle (figure 2*c*).

Figure 3 illustrates the patterns of expression of the SIPC protein in the stage 2 nauplius and the cyprid. In the nauplius (figure 3*a*), the SIPC protein distribution was similar to that of the SIPC mRNA (figure 2*a*). In the cyprid, the expressed SIPC was localized in the epidermis and the cuticle, for example, in the cuticle of the bivalved

carapace (figure 3*b*,*c* and *e*) and the thoracopods (figure 3*b*), and in the cuticle surrounding the compound eyes (figure 3*d*). Figure 3 is representative of the majority of cyprids. The controls did not show any specific staining (figure 2 in electronic supplementary material).

(d) *Distribution of the settlement-inducing protein complex mRNA and the expressed settlement-inducing protein complex in adult* B. amphitrite

The distributions of the SIPC mRNA and expressed SIPC in adult *B. amphitrite* are shown in figure 4. As for the larvae (figure 2), the SIPC mRNA was restricted to the cuticle, for example that of the cirri (figure 4*a*), and the penis (figure 4*b*). The expressed SIPC also showed a similar distribution, for example, in the cuticle of the cirri (figure 4*c*), the penis (figure 4*d*,*e*) and in the hindgut (figure 4*f*). Both the old and the new cuticle were stained positively (figure 4*e*). No specific staining was evident in the controls (figure 3 in electronic supplementary material). The basic Masson's trichrome staining, which indicates the distribution of basophilic structures such as the cuticle, is shown in figure 3 in electronic supplementary material.

4. DISCUSSION

Knight-Jones (1953) was the first to suggest that the barnacle settlement cue might be a cuticular protein based on evidence from bioassay studies and physicochemical similarities with the insect cuticular protein 'arthropodin'. Larman *et al.* (1982) more recently demonstrated that the settlement factor was present in the adult barnacle cirri and the soft body. We have now clearly demonstrated, for the first time, that the settlement cue, known in *B. amphitrite* as the SIPC (Matsumura *et al.* 1998*a*), is a cuticular glycoprotein.

In arthropods the cuticle is composed of the procuticle (inner tegument) and the epicuticle (outer tegument; Compère *et al.* 2004; Willis *et al.* 2005). In this study, the SIPC mRNA and expressed protein were first detected in the stage 2 nauplius by *in situ* hybridization and immunohistochemistry, where it was expressed most strongly in the segmented limbs (paired antennules, antennae and mandibles), the labrum and the hindgut (figure 2*a*) and in the stage 6 nauplius by western blotting (figure 1*b*; see Walley 1969 for larval morphology). The inability to detect the SIPC in earlier naupliar stages by western blotting may reflect the limit of sensitivity of our immunoblotting as we have previously detected the SIPC transcript in all larval stages by RT-PCR (Dreanno *et al.* in revision). In subsequent developmental stages the SIPC was found in all organs with cuticular tissue; it appeared in the cuticle of the thoracopods, compound eyes and bivalved carapace (cyprid), the hindgut (cyprid and adult), and in the cirri and penis (adult only). Variability in immunohistochemical staining of the cyprid may reflect the relatively low permeability of the cyprid bivalved carapace to antibodies. The absence of the SIPC from the midgut and muscle tissues is particularly relevant as *neither* of these tissues contains cuticular proteins (Rainbow & Walker 1977). The presence of the SIPC in the haemolymph might be explained *either* by a contamination of our samples by epidermis or ovary, which *both* occur at the base of the body, or it may be due to the presence of cuticular proteins produced by the epidermis and secreted into the haemolymph (Saas *et al.* 1993). The pattern of SIPC expression we observed during development (figure 1*b*) and reported previously (Matsumura *et al.* 1998*b*; Dreanno *et al.* 2004) could now be explained simply by the increase in cuticular mass from the nauplius to the adult.

The arthropod cuticle was considered traditionally to be an inert supporting exoskeleton, a protective barrier against microorganisms and an exchange surface (Adachi *et al.* 2005). However, recent evidence suggests that the arthropod cuticle also contributes to various biochemical and physiological processes (Willis 1999; Willis *et al.* 2005). Although the SIPC is present in *both* old and new cuticle of *B. amphitrite* (figure 4*e*), we believe it is unlikely that it is a structural protein since it does not contain the R&R (Rebers & Riddiford 1988) consensus sites that are involved in binding cuticular proteins to chitin (Rebers & Willis 2001; Willis *et al.* 2005). A particular role of the arthropod cuticle is now realized to be in the immune response since it contains serine proteinases (Buda & Shafer 2005). In this respect, it is interesting to note that the *B. amphitrite* SIPC shows sequence similarities to the α_2-macroglobulin (A2M) protein family (Dreanno *et al.* in revision), which is involved in innate immunity (Armstrong & Quigley 1999) and that in the lobster, an A2M-like protein may play a role in olfaction (Hollins *et al.* 2003). Moreover, a remarkable parallel to the SIPC exists in the copepod, *Tigriopus japonicus*, where a 70 kDa protein, bearing some sequence similarity to A2M, has been implicated in mate recognition via a contact chemical sense (Ting & Snell 2003).

We now regard the SIPC as a contact pheromone involved in gregarious settlement behaviour. The localization of this cue to cuticle means that one mode of detection could be through contact of the cyprid (presumably with the paired antennules) with the cuticle that covers the basal region of the shell plates of the adult barnacle (Bourget 1977). Moreover, we have shown previously that the attachment disc of the cyprid antennules immunostains for the SIPC and that this glycoprotein is deposited on the substratum during the cyprid 'exploratory' walk prior to final settlement (Matsumura *et al.* 1998*b*; Dreanno *et al.* 2006); these 'footprints' then act as a cue to other cyprids promoting additional settlement (Yule & Walker 1985; Clare *et al.* 1994). The localization of the SIPC in the barnacle cuticle suggests that in addition to functioning as a contact pheromone, this glycoprotein might also act as a waterborne cue (an attractant?) if it were released into the environment. Indeed, Harrison (1998) detected settlement-inducing activity in seawater that had been in contact with crude extracts of barnacle tissues that would have contained the SIPC. Being a water-soluble glycoprotein (Crisp & Meadows 1962), the SIPC could be released during moulting or cuticle regeneration, but a more likely means would be through bacterial action. Hermit crabs, for example, detect molluscan shells by peptide cues—kairomones—that are released from the shell by serine proteolytic hydrolysis of structural proteins (Rittschof & Cohen 2004). Bacteria may therefore play a role in barnacle settlement by degrading the SIPC into peptide fragments that can be detected in the water column by cypris larvae; this concept of bacteria

functioning in odour generation is not new (Penn & Potts 1998). Significantly, specific small peptides are able to induce barnacle settlement (cf. Clare & Yamazaki 2000; Browne & Zimmer 2001).

There is strong evidence for crustacean contact pheromones (Snell *et al.* 1995; Frey *et al.* 1998; Kelly *et al.* 1998; Cornette *et al.* 2002; Ting & Snell 2003; Pasternak *et al.* 2004) of which the *B. amphitrite* SIPC is now the most completely characterized (Dreanno *et al.* in revision). Since *both* the SIPC-N and SIPC-C antibodies detected the SIPC protein in the barnacle cuticle and its distribution were similar to that of the SIPC mRNA, there appears to be no differential localization of the SIPC subunits that are seen on SDS-PAGE (Matsumura *et al.* 1998*b*), which reinforces our belief that the SIPC is a single cuticular glycoprotein. Cuticular proteins comprise one of the largest multigene families in arthropods (Magkrioti *et al.* 2004) and different anatomical regions show different histological and cuticular protein composition (Willis 1999; Willis *et al.* 2005). Further studies on the ultrastructural localization of the SIPC in barnacle cuticle are now required, together with a focus on the nature of the SIPC receptor and its location. Progress towards those goals will help our understanding of the evolution of gregarious settlement behaviour and we hope it may play an important role in the development of new ecologically-sensitive antifouling technologies.

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